JOURNAL OF SURGICAL RESEARCH 198 (2015) 515–524

Available online at www.sciencedirect.com

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Effect of limb demand ischemia on autophagy and morphology in mice

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ARTICLE INFO

Article history:
Received 2 January 2015
Received in revised form
19 February 2015
Accepted 2 April 2015
Available online 9 April 2015

Keywords:
Limb demand ischemia
Diet-induced obesity
Type-2 diabetes
Peripheral arterial disease
Autophagy
Mitochondrial biogenesis
Muscle regeneration

ABSTRACT

Background: Obesity is a major risk factor for diabetes and peripheral arterial disease, which frequently leads to lower limb demand ischemia. Skeletal muscle autophagy and mitochondrial biogenesis are important processes for proper oxidative capacity and energy metabolism, which are compromised in diabetes. This study compares autophagy, mitochondrial biogenesis, energy metabolism, and morphology in the hind limbs of obese diabetic mice subjected to demand or sedentary ischemia.

Materials and methods: Unilateral hind limb demand ischemia was created in a group of diet-induced obese mice after femoral artery ligation and 4 wk of daily exercise. A parallel group of mice underwent femoral artery ligation but remained sedentary for 4 wk. Hind limb muscles were analyzed for markers of autophagy, mitochondrial biogenesis, adenosine triphosphate, and muscle tissue morphology.

Results: At the end of the 4-wk exercise period, demand ischemia increased the autophagy mediator Beclin-1, but it did not alter the autophagy indicator, LC3B-II/I ratio, or markers of mitochondrial biogenesis, optic atrophy/dynamin-related protein. In contrast, exercise significantly increased the level of mitochondrial protein-succinate dehydrogenase subunit-A and reduced adipocyte accumulation and the percentage of centrally nucleated myofibers in the demand ischemia limb. In addition, demand ischemia resulted in decreased uncoupling protein-3 levels without altering muscle adenosine triphosphate or pS473-Akt levels.

Conclusions: Limb demand ischemia markedly decreased adipocyte accumulation and enhanced muscle regeneration in obese mice, but it did not appear to enhance autophagy, mitochondrial biogenesis, energy metabolism, or insulin sensitivity. Future studies aimed at evaluating novel therapies that enhance autophagy and mitochondrial biogenesis in diabetes with peripheral arterial disease are warranted.

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0022-4804/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2015.04.008

1. Introduction

Obesity and the metabolic syndrome predispose patients to the development of diabetes and peripheral arterial disease (PAD). In obese patients, demand ischemia-induced claudication worsens their prognosis, independent from other confounding factors [1,2]. Pathologically, demand ischemia in patients with PAD causes repetitive cycles of ischemic injury that exacerbate mitochondrial dysfunction, which induces oxidative stress [3], and gait deficits [4]. Similar to muscle specimens from PAD patients, mice with chronic hind limb ischemia display evidence of myopathy associated with mitochondrial dysfunction and oxidative damage [5]. A correlation has been established between the severity of PAD, diminished lower limb skeletal muscle protein levels, and mitochondrial content in the diseased limb [6]. Histologic studies characterizing the morphology of skeletal muscle specimens of PAD patients [7,8] have revealed evidence of myofiber degeneration with variable levels of fibrosis and fatty deposition [9].

The etiology of type-2 diabetes, a major risk factor for PAD, has been linked with skeletal muscle insulin resistance, but the exact mechanism requires further investigation. Substantial evidence has implicated increased oxidative stress, mitochondrial dysfunction, and intramuscular fat accumulation as the primary targets [10]. Recently, research has focused on investigating the role of autophagy and defective mitochondrial biogenesis in the pathogenesis of insulin resistance in ageing [11] and in the metabolic syndrome with the associated comorbidities in cardiovascular disease [12,13]. Autophagy and mitochondrial biogenesis are synchronized processes important in the regulation of energy metabolism and cellular homeostasis that also provide tissue protective mechanism under stress or pathologic conditions [14]. Consisting of several cellular degradation pathways, autophagy targets partially degraded or aged cytoplasmic materials or organelles including mitochondria for lysosomal enzymatic digestion and recycling. Mitochondrial biogenesis consists of mitochondrial fusion and fission, which are particularly important in bioenergetics, tissue remodeling, and function of skeletal muscle [15].

To date, limited literature is available on the effects of exercise on limb muscle autophagy and morphology after demand ischemia in the setting of type-2 diabetes. We hypothesized that the pattern of molecular responses associated with autophagy, mitochondrial biogenesis, energy metabolism, and muscle morphology would be different in demand ischemia compared with sedentary ischemia.

Therefore, the purpose of this study was to evaluate the expression of selected regulatory proteins involved in autophagy and mitochondrial dynamics in diet-induced obese diabetic mice subjected to demand or sedentary ischemia after a 4-wk exercise or sedentary period. This study also aimed to elucidate the effect of a prolonged period of daily exercise on energy substrate levels, insulin sensitization, and morphologic indices of skeletal muscle regeneration, including pathologic adipocyte accumulation.

2. Materials and methods

2.1. Animal protocol

Animal care and experimental procedures were in compliance with the "Principal of Laboratory Animal Care" (Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 86-23, 1985) and approved by the institutional review committee. Age-matched C57BL6 male mice were acquired from Jackson Laboratory (Bar Harbor, ME) after being fed a diet consisting of 60% kcal from fat for 26 wk to induce obesity, insulin resistance, and diabetes [16]. To induce hind limb ischemia, all mice were subjected to unilateral femoral artery ligation (FAL) surgery in one hind limb or sham surgery in the contralateral hind limb and then were allowed to recover for 2 wk as previously described [17,18]. Subsequently, the mice were divided into two groups: exercise group and sedentary group. The exercise group ($n = 8$) underwent 60 min of daily treadmill exercise (12 m/min speed at 10° incline) for 4 wk to induce demand ischemia condition in the hind limb that underwent FAL and exercised nonischemic contralateral hind limb in each mouse. The sedentary group ($n = 7$) was not exercised for 4 wk after the recovery period after FAL thus creating the sedentary ischemia in the hind limb that had FAL and sedentary nonischemic contralateral hind limb in each animal. The activity of the sedentary mice was not restricted in the cage. Whole body weight and hind limb laser Doppler imaging were documented at baseline before surgery and once a week thereafter. Hind limb muscle tissues were harvested at the end of the 4-wk exercise or sedentary period. Collected tissues were divided then either fixed in paraformaldehyde for histologic evaluation or stored at −80°C for molecular analyses.

2.2. SDS-Polyacrylamide Gel Electrophoresis immunoblotting for markers of autophagy, mitochondrial biogenesis, phosphorylated-Akt, and uncoupling protein-3

100 micrograms soluble protein aliquots isolated from hind limb tissue using RIPA extraction buffer were subjected to Tris-HCl-SDS electrophoresis and electro-blotting transfer followed by chemiluminescence detection as previously described [16]. To detect protein markers of autophagy, the membranes were incubated with an IgG against the microtubule-associated protein light chain-3B (LC3B; Cell Signaling, Danvers, MA) and rabbit monoclonal anti–Beclin-1 (Cell Signaling). To assess markers of mitochondrial biogenesis, membranes were incubated with rabbit polyclonal anti–optic atrophy-1 (Opa-1) IgG (Millipore, Billerica, MA) and rabbit monoclonal anti–dynamin-related protein-1 (Drp-1) IgG (Cell Signaling). To evaluate the mitochondrial protein content, membranes were probed with mouse antibody that recognizes the mitochondrial complex II protein: succinate dehydrogenase subunit-A (SDHA; Abcam, Cambridge, MA) or rabbit polyclonal anti–cytochrome-c IgG (Cell Signaling). To evaluate the PI3/Akt pathway activity, we assessed the level of phosphorylated-Akt, using rabbit monoclonal IgG that

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